ACYL ADENYLATES: THE INTERACTION OF ADENOSINE TRIPHOSPHATE AND L-METHIONINE*

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(Received for publication, March 9, 1956)

Several reactions are now known which can account for the exchange of PP[1] with the terminal pyrophosphate group of ATP (1–6). Recently it was demonstrated (3, 4) that the reaction of ATP and acetate with the acetate-activating enzyme (aceto-CoA-kinase) resulted in such an exchange. Because of this and other observations (4), it was proposed that the acetate-dependent exchange occurred via the intermediate formation of adenyl acetate (Reaction 1).

(1) $$\text{ATP} + \text{acetate} \rightleftharpoons \text{adenyl acetate} + \text{PP}$$

Further investigation of PP-ATP exchange reactions revealed that yeast extract catalyzed such an exchange, which was dependent on the presence of L-methionine (3).

The possibility that this represented an activation of methionine by ATP, analogous to that shown in Reaction 1, stimulated further study of its mechanism. The present report is concerned with the partial purification of an enzyme from yeast which carries out the L-methionine-dependent exchange of $P^{32}P^{32}$ and ATP, and a description of some of the properties of the reaction. In the presence of ATP, L-methionine, and hydroxylamine there is a net formation of A5P, PP, and methionine hydroxamic acid. These findings, together with the failure to observe any exchange of A5P-C^{14} with ATP in the presence of methionine, are consistent with the formation of an adenyl methionine derivative from ATP and methionine (Reaction 2) and its subsequent cleavage in the presence of hydroxylamine (Reaction 3).

(2) $$\text{ATP} + \text{methionine} \rightleftharpoons \text{adenyl methionine} + \text{PP}$$

(3) $$\text{Adenyl methionine} + \text{hydroxylamine} \rightarrow \text{methionine hydroxamate} + \text{A5P} + \text{PP}$$

* This work was supported by grants from the United States Public Health Service and the National Science Foundation.

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[1] The following abbreviations have been used. PP, inorganic pyrophosphate; ATP or ARPPP, adenosine triphosphate; A5P, adenosine-5′-phosphate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; CoA, coenzyme A.

Hoagland (5) has reported the existence of amino acid-requiring PP-ATP exchange reactions in liver extracts and demonstrated the formation of amino acid hydroxamates from ATP, amino acids, hydroxylamine, and a soluble protein fraction from liver. DeMoss and Novelli (6) have also demonstrated the existence of a number of amino acid-dependent PP-ATP exchange reactions in a variety of bacterial extracts. More recently Hoagland *et al.* (7) reported the separation of the enzymatic activity responsible for the methionine-activated PP-ATP exchange reaction from other amino acid-requiring PP-ATP exchange systems and have suggested that each of the exchange reactions is catalyzed by a separate enzyme.

Materials and Methods

$P^{32}P^{32}$ was prepared by heating $Na_2HP^{32}O_4$ at 225° for 18 hours (8), or at 400° for 1 hour (9), and purified by anion exchange chromatography (8). ATP labeled with P^{32} in the terminal pyrophosphate group was prepared by exchange of $P^{32}P^{32}$ with ATP by using purified aceto-CoA-kinase (4). A5P-C^{14} was made as previously described (4). A5P deaminase was obtained from rabbit muscle by the method of Kalckar (10), and crystalline ribonuclease was obtained from the Worthington Biochemical Corporation. Hydroxylamine was prepared from hydroxylamine sulfate and barium hydroxide (4), and DL-methionine hydroxamate[2] was made by treatment of DL-methionine hydrochloride ethyl ester (11) with methanolic hydroxylamine in sodium methoxide (12) and recrystallized three times from methanol-water mixtures at about pH 8. Methionine hydroxamic acid was determined colorimetrically as the ferric complex (13). To the sample in a volume of 1 ml. was added 0.5 ml. of a 10 per cent solution of ferric chloride containing 0.2 M TCA and 0.66 M HCl. After 5 minutes the optical density at 540 mμ was determined in a cuvette with a 1 cm. light path. 1 μmole of methionine hydroxamic acid gave, under these conditions, an optical density of 0.437. In the experiments in which the enzymatic formation of methionine hydroxamate was measured, synthetic methionine hydroxamate was added to a control tube (minus ATP and methionine) as an internal standard.

A5P was determined either by anion exchange chromatography (14) or by A5P deaminase (15), and PP was measured by phosphate liberation (16) after treatment with inorganic pyrophosphatase[3] (17). Protein was determined as described by Lowry *et al.* (18).

[2] I am deeply indebted to Dr. Peter H. Lowy, to Dr. E. B. Keller, and Dr. M. B. Hoagland for their generous gifts of DL-methionine hydroxamate which were used for comparison with the preparation described above.

[3] The crystalline inorganic pyrophosphatase, prepared from yeast, was very kindly supplied by Dr. G. Perlmann and Dr. M. Kunitz.

Assay Procedure—The methionine-dependent $P^{32}P^{32}$-ATP exchange reaction was measured in the following way. The reaction mixture contained, in 1.0 ml., 0.1 M Tris buffer, pH 8.0, 0.002 M ATP, 0.002 M $P^{32}P^{32}$ containing between 10^4 and 10^5 c.p.m. per μmole, 0.003 M L-methionine, 0.005 M $MgCl_2$, and the enzyme. After 15 minutes at 37°, perchloric acid was added and the P^{32} incorporated into ATP was measured by adsorption and elution of the ATP from Norit (4). 1 unit of activity was defined as that amount of enzyme which catalyzed the incorporation of 1 μmole of $P^{32}P^{32}$ into ATP in 15 minutes. With 0.3 unit of enzyme or less, the rate of exchange was constant for at least 30 minutes. By the standard assay, the amount of exchange was proportional to enzyme concentration. Thus, with 1.5, 3.0, 5.0, 12.5, and 25 γ of enzyme protein (Fraction AS-1) the number of units of activity per mg. of protein were 6.7, 6.7, 6.0, 5.6, and 6.4.

Results

Dried brewers' yeast[4] (25 gm.) was mixed with 75 ml. of 0.1 M potassium bicarbonate and incubated at 37° for 4 hours. The autolyzed mixture was centrifuged at 10,000 × *g* for 10 minutes and the residue discarded. The supernatant fluid (yeast extract (Fraction YE) 40 ml.) was diluted to 120 ml. with cold water, and 76 ml. of ethanol at 4° were added at a rate adjusted to maintain the temperature between 7–10°. The solution was centrifuged at 4° for 5 minutes at 10,000 × *g* and the supernatant fluid discarded. The precipitate was extracted with 54 ml. of 0.05 M Tris buffer, pH 8.0, and the insoluble material obtained by centrifugation was discarded. To the supernatant fluid were added 27 ml. of 0.5 M potassium succinate buffer, pH 6.0, the solution was cooled to 0°, and 23.5 ml. of ethanol at −15° were added while the temperature was kept between 0–1°. The precipitate was removed by centrifugation for 5 minutes at 10,000 × *g* and then dissolved in 40 ml. of 0.05 M Tris buffer, pH 8.0 (Alcohol Fraction 2).

To this solution were added 13.4 gm. of ammonium sulfate and, after 5 minutes, the mixture was centrifuged as mentioned above and the precipitate discarded. To the supernatant fluid were added 5.0 gm. of ammonium sulfate, and, after 5 minutes, the solution was centrifuged. The precipitate was dissolved in 10 ml. of Tris buffer, pH 8.0 (Ammonium sulfate, Fraction 1 (AS-1)).

This solution was then diluted with the Tris buffer to a protein concentration of 2.0 to 2.5 mg. per ml. In the experiment in Table I, the volume was adjusted to 17.5 ml. and, after being warmed to 20°, 1.5 mg. of crystalline ribonuclease in 0.3 ml. of water were added. After 5 minutes the solu-

[4] Dried brewers' yeast, strain BSC, was kindly furnished by Anheuser-Busch, Inc., St. Louis.

tion was cooled to 0° and 31 ml. of cold saturated ammonium sulfate were added. After another 5 minutes the precipitate was removed by centrifugation for 10 minutes at 10,000 × *g*. To the supernatant fluid were added 20 ml. of saturated ammonium sulfate, and after 5 minutes the precipitate was centrifuged, as described above, and dissolved in 6 ml. of 0.05 M Tris buffer, pH 8.0 (Ammonium sulfate, Fraction 2 (AS-2)).

TABLE I

Purification of Enzyme

Fraction	Concentration of enzyme	Total units	Concentration of protein	Specific activity
	units per ml.		*mg. per ml.*	*units per mg. protein*
Yeast extract (YE)	16.9	676	50.7	0.33
Alcohol fraction 2 (A-2)	15.2	608	5.0	3.0
Ammonium sulfate Fraction 1 (AS-1)	34.0	340	4.1	8.3
" " " 2 (AS-2)	28.4	170	1.9	15.0

TABLE II

Requirements for Exchange of $P^{32}P^{32}$ with ATP

Components	$P^{32}P^{32}$ incorporated into ATP
	μmole
Complete	0.38
No ATP	0.01
" methionine	0.03
" $MgCl_2$	0.01
" enzyme	0.00

The conditions were the same as those described for the assay of the enzyme. 50 γ of enzyme Fraction AS-1, specific activity 7.5, were used.

Fraction AS-2 lost about 30 per cent of its initial activity in 1 week when stored at −15°. In all of the experiments reported here, Fraction AS-1, which was more stable, was used. Fraction AS-1 still contained some ATP-splitting activity but no detectable inorganic pyrophosphatase (17) or adenylic kinase (19).

Requirements for PP-ATP Exchange—In the crude yeast extract there was little or no increase in the rate of the exchange reaction upon the addition of methionine. With the purified fractions (Ammonium sulfate, Fractions 1 and 2) little or no exchange of PP and ATP occurred unless L-methionine and Mg^{++} were added (Table II). Most preparations of the enzyme still contained some activity in the absence of added methionine,

but this rarely exceeded 10 per cent of the rate obtained in the presence of optimal amounts of methionine.

The amount of methionine necessary to promote the maximal rate of exchange was 1×10^{-4} M, and for half maximal rate it was 1×10^{-5} M (Table III). It might be pointed out that this procedure offers a relatively simple and rapid method for detecting and determining small amounts of L-methionine.

Specificity of L-Methionine Requirement—Of the naturally occurring amino acids, only methionine catalyzed a significant amount of exchange of $P^{32}P^{32}$ and ATP with Fraction AS-1 (Table IV). Other amino acids alone or in various combinations gave values no higher than the control

TABLE III

Effect of L-Methionine Concentration on Rate of PP-ATP Exchange Reaction

Concentration of L-methionine	$P^{32}P^{32}$ incorporated into ATP
$\times 10^4$ M	μmole
0	0.04
0.03	0.11
0.06	0.17
0.10	0.19
0.30	0.30
1.0	0.39
3.0	0.39
7.0	0.39
15	0.41

The conditions were the same as those described for the assay of the enzyme. 50 γ of enzyme Fraction AS-1, specific activity 7.5, were used.

with nothing added. Furthermore, other amino acids, alone or in combination, did not inhibit the methionine-activated exchange. The requirement for methionine was found to be specific for the L form. D-Methionine was inactive, and did not inhibit the effect of L-methionine when both were present at equal concentrations (1×10^{-3} M). Methionine sulfoxide, methionine sulfone, and homocystine were also inactive. The only other amino acid which has been found to promote the exchange reaction was DL-ethionine, but, because extremely small amounts of methionine are active, the exchange found with ethionine may be due to contamination with methionine.

Formation of Methionine Hydroxamic Acid—Because the methionine-activated PP-ATP exchange reaction appeared to be somewhat analogous to the acetate-dependent exchange reaction by aceto-CoA-kinase (3, 4), experiments were carried out to detect the enzymatic formation of methionine

hydroxamate in the presence of hydroxylamine. Incubation of ATP, methionine, Mg^{++}, hydroxylamine, and the enzyme resulted in the formation of equivalent amounts of methionine hydroxamic acid, A5P, and PP when the values in the absence of methionine were subtracted (Table V). Under these conditions, there was an almost linear rate of methionine

TABLE IV

Effect of Various Amino Acids on PP-ATP Exchange

Amino acid		$P^{32}P^{32}$ incorporated into ATP
		μmole
None		0.01
L-Methionine	1×10^{-3} M	0.13
L-Tryptophan	1×10^{-3} "	0.01
DL-Alanine	1×10^{-3} "	0.01
L-Histidine	1×10^{-3} "	0.02
L-Glutamic acid	1×10^{-3} "	0.01
L-Isoleucine	1×10^{-3} "	0.01
DL-Serine	1×10^{-3} "	0.00
L-Phenylalanine	1×10^{-3} "	0.01
L-Valine	1×10^{-3} "	0.02
L-Threonine	1×10^{-3} "	0.02
L-Leucine	1×10^{-3} "	0.01
L-Proline	1×10^{-3} "	0.01
DL-Homocysteine	1×10^{-3} "	0.01
Glycine	1×10^{-3} "	0.01
L-Tyrosine	1×10^{-3} "	0.02
None		0.04
L-Methionine	1.5×10^{-3} M	0.41
D-Methionine	1×10^{-3} "	0.05
"	3×10^{-3} "	0.04
DL-Ethionine	3×10^{-3} "	0.12

Both experiments were carried out as described for the usual assay procedure. In the first experiment, 12 γ of enzyme Fraction AS-1, specific activity 10, were used, and in the second experiment 50 γ of Fraction AS-1, specific activity 7.5.

hydroxamic acid and PP formation. However, this rate decreased rapidly after 60 minutes and was not restored by the addition of more ATP and methionine. The reason for this is not clear.

When the enzyme, ATP, Mg^{++}, or methionine was omitted, there was no significant formation of methionine hydroxamic acid above that observed in the absence of methionine. The absorption spectrum of the enzymatically produced methionine hydroxamate in the presence of ferric chloride at acid pH was characteristic of acyl hydroxamic acids. This spectrum exhibited a broad maximum between 495 and 510 mμ.

Nature of Reaction of ATP and Methionine—Two possible interpretations of the mechanism of the reaction were considered. In the first, ATP and methionine react to form a methionine pyrophosphate compound which can subsequently exchange the bound pyrophosphate group for free $P^{32}P^{32}$ (Reactions 4 and 5). In the second, the products formed are adenyl methionine and free PP (Reaction 6), which by reversal of the reaction converts $P^{32}P^{32}$ to ATP^{32}.

TABLE V

Enzymatic Formation of Methionine Hydroxamate, A5P, and PP from ATP, Methionine, and Hydroxylamine

The values are given in micromoles per ml.

Experiment No.	Time	Additions	A5P	Δ*	PP	Δ*	Methionine hydroxamate	Δ*
	min.							
1	30	Complete			1.47	+1.33	1.24	+1.19
	60	"			2.17	+1.90	2.00	+1.93
	30	No methionine			0.14		0.05	
	60	" "			0.27		0.07	
2	30	Complete	1.79	+1.63	1.81	+1.72	0.97	+0.88
	30	No methionine	0.95	+0.79	0.99	+0.90	0.09	0.00
	30	" ATP	0.16		0.09		0.09	

* The difference was calculated by subtracting the values obtained in the absence of either methione or ATP.

Experiment 1. The reaction mixture (1.0 ml.) contained 0.10 M Tris buffer, pH 8.0, 0.005 M $MgCl_2$, 0.011 M ATP, 0.01 M L-methionine, 2.5 M hydroxylamine, and 1.2 mg. of Fraction AS-1, specific activity 12.1. After incubation at 37°, aliquots were removed for measurement of methionine hydroxamic acid formation as previously described, and PP was determined after conversion to inorganic phosphate with inorganic pyrophosphatase. Experiment 2. The reaction mixture (1.15 ml.) contained 0.09 M Tris buffer, pH 8.0, 0.004 M $MgCl_2$, 0.011 M ATP^{32} containing 5400 c.p.m. per μmole, 0.01 M L-methionine, 2.2 M hydroxylamine, and 1.2 mg. of Fraction AS-1, specific activity 11.4. After incubation at 37°, aliquots were measured for methionine hydroxamate as described previously. One series of aliquots was acidified and treated with Norit (4), and the PP was calculated from the P^{32} in the nucleotide-free supernatant fluid. Separate aliquots were chromatographed on Dowex 1 to separate the A5P, and the total amount of A5P was determined from the optical density at 260 mμ.

(4) $$\text{ATP} + \text{L-methionine} \rightleftharpoons \text{L-methionine-PP} + \text{A5P}$$

(5) $$\text{L-Methionine-PP} + P^{32}P^{32} \rightleftharpoons \text{L-methionine-}P^{32}P^{32} + \text{PP}$$

(6) $$\text{ATP} + \text{L-methionine} \rightleftharpoons \text{adenyl L-methionine} + \text{PP}$$

To distinguish between these two possibilities, the exchange of A5P-C^{14}

and ATP was studied. By mechanism (1), A5P-C^{14} should exchange with ATP at least as rapidly as does $P^{32}P^{32}$, and this should require L-methionine. By mechanism (2), there should be no exchange. It was found (Table VI)

TABLE VI

Exchange of A5P-C^{14} and ATP

Components	Specific activity of compound isolated	
	A5P*	ATP*
	c.p.m. per μmole	*c.p.m. per mole*
Complete	15,000	<100
No methionine	15,500	<100
" enzyme	15,000	<100

* No correction for the self-absorption due to the salt of the eluate was made.

The reaction mixture contained in 1 ml. of 0.14 M Tris buffer, pH 8.0, 0.005 M $MgCl_2$, 0.001 M ATP, 0.001 M A5P-C^{14} containing 2.25×10^4 c.p.m. per μmole, and 17 γ of Fraction AS-1, specific activity 6.7. Time 30 minutes; temperature 37°. At the end of the incubation, A5P and ATP were separated by anion exchange chromatography (14) and the radioactivity of an aliquot of the eluate from the peak tube was determined.

TABLE VII

Formation of PP from ATP

Labeled substrate	Time	P^{32} incorporated into PP		Δ	P^{32} incorporated into ATP		Δ
		Plus methionine	Minus methionine		Plus methionine	Minus methionine	
	min.	*μmole*	*μmole*		*μmole*	*μmole*	
$ARPP^{32}P^{32}$	15	0.099	0.028	+0.071			
	30	0.191	0.060	+0.131			
$P^{32}P^{32}$	15				0.089	0.016	+0.073
	30				0.157	0.020	+0.137

The reaction mixture contained, in 1 ml., 0.16 M Tris buffer, pH 8.0; 0.005 M $MgCl_2$; 0.001 M ATP or ATP^{32} ($ARPP^{32}P^{32}$) containing 4.35×10^4 c.p.m. per μmole; 0.005 M L-methionine; 0.001 M PP or $P^{32}P^{32}$ containing 3.28×10^4 c.p.m. per μmole; 15 γ of Fraction AS-1, specific activity 4.8. Temperature 37°. P^{32} incorporated into ATP was determined as in the usual assay procedure and P^{32} into PP was measured in the supernatant fluid after adsorption of the ATP^{32} with Norit.

that there was little or no incorporation of A5P-C^{14} into ATP either in the presence or absence of L-methionine. In a comparable experiment in which 1 μmole of $P^{32}P^{32}$ replaced the A5P-C^{14}, there was an incorporation of 0.22 μmole of PP into ATP, or about 44 per cent of the maximal exchange possible. This evidence would then appear to exclude the first hypothesis shown in Reactions 4 and 5.

Several attempts have been made to demonstrate a net formation of PP from ATP in the presence of L-methionine and in the absence of hydroxylamine, but these have been unsuccessful to date. ATP labeled with P^{32} in the pyrophosphate group was incubated with the enzyme, Mg^{++}, and inorganic pyrophosphatase in the presence and absence of L-methionine and P^{32} inorganic phosphate was measured in the Norit-non-adsorbable fraction. There was no significant difference in the amount of P^{32} liberated in the presence or absence of L-methionine. However, if in place of the inorganic pyrophosphatase a pool of unlabeled PP was added, considerable radioactivity from the ATP was trapped in the PP and could be determined in the Norit-non-adsorbable fraction (Table VII). The pyrophosphate liberated was equivalent to the amount of $P^{32}P^{32}$ incorporated into ATP in a parallel experiment with $P^{32}P^{32}$ and unlabeled ATP.

Thus it is clear that, while the enzyme catalyzes the formation of free PP, the reaction does not appear to proceed very far in this direction. Whether this is due to the formation of an enzyme-bound, adenyl methionine compound, as had been previously suggested (5, 7), remains to be established.

DISCUSSION

The results obtained in the present work with L-methionine show certain similarities to those obtained with the acetate-activating system (aceto-CoA-kinase) from yeast. In both there is a reaction of ATP with a compound containing an acyl group, which results in the exchange of the pyrophosphoryl group of ATP with free PP. The A5P moiety of the ATP remains in a "bound" form; that is, not exchangeable with free A5P. Furthermore, in both systems the formation of any product of the reaction, except by exchange reactions or by the use of high concentrations of hydroxylamine, has not been demonstrated. The formation of methionine hydroxamate suggests that the methionine is linked through the carboxyl group, presumably to the phosphate group of A5P.

One notable difference between the acetate-activating system and the present reaction with methionine is the subsequent transfer of the acetyl group to an acceptor (CoA). This poses the question of what is the natural acceptor of the methionine moiety. Several attempts were made to detect an acceptor in crude yeast extracts by measuring PP liberation from ATP in the presence of the enzyme, methionine, and crude yeast fractions. There was, however, no formation of PP which could not be accounted for by the activity in the crude extracts of ATPase. Hoagland *et al.* (7) have suggested the possibility that the "protein-forming site" functions as the acceptor and that the amino acids activated in this way are linked together to form the polypeptide structure. Maas (20), in his studies of pantothenic acid synthesis, has proposed that an adenyl pantoate derivative is formed

from ATP and pantoic acid and that the pantoyl group is transferred to the acceptor amine β-alanine. The question of whether adenyl amino acids are enzymatically utilized for protein formation in the absence of an energy source requires further work, however.

SUMMARY

An enzyme has been isolated from yeast which catalyzes an L-methionine-dependent exchange of $P^{32}P^{32}$ with ATP. D-Methionine and other amino acids alone or in combination do not replace the function of L-methionine. In the presence of ATP, L-methionine hydroxylamine, and the enzyme, there is a net formation of methionine hydroxamic acid, A5P, and PP. This, plus the fact that A5P-C^{14} does not exchange with ATP, suggests that the PP-ATP exchange can be accounted for by the following reaction:

$$\text{ATP} + \text{L-methionine} \rightleftharpoons \text{adenyl L-methionine} + \text{PP}$$

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